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ENUMERATION, ISOLATION AND IDENTIFICATION PHENOTYPIC THERMOPHILE LACTIC ACID BACTERIA ISOLATED FROM DIFFERENT FERMENTED MILK COLLECTED IN ALGERIA

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ABSTRACT

The lactic acid bacteria which are considered the most useful microorganism to society are involved in many manufacture fermented foods such as yogurt and chees. The purpose of this study was to isolate and characterize the thermophile lactic acid bacteria from different fermented milk collated in Algeria. twenty samples of different milk used for this experiment were obtained from camel in Adrar province and goat, sheep, cow in Relizane area in Algeria. A total of 50 colonies were grown in MRS acetic and LM17 agar. The pre-identification tests were performed according to the morphological characteristics such as catalas, Gram, growth at 10C°, growth in presence 6.5%NaCl. The isolates were Subjected to different screening test and identified as presumptive lactic acid bacteria and classified to the genera *Lactobacillus* (17), *Streptococcus* (04), *Enterococcus*(29). The isolated species lactic acid bacteria using API50CHL, API20 Strep were *Lactobacillus delbruckiibulgaricus* (10%), *Streptococcus thermophilus* (8%) and *Enterococcus faecuim* (34%) *Enterococcus faecalis* (24%). Among of the lactic acid bacteria, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* produced The high among acid compared with other lactic acid bacteria. the strains showed properties suggest that thyare good candidate for dairy fermentation process.

KEYWORDS: Algeria, Fermented Milk, Lactic Acid Bacteria, Proprieties Technologic

INTRODUCTION

Lactic acid bacteria (LAB) are indigenes habitants in different drily product. It has been long consumed by people in several fermented food. and widely used for fermentation and preservation of a wide range of milk, meat and vegetable foods **(Zhu, Liu, & Wu, 2000).** The mechanism of fermentation the dairy products is metabolize the lactose to lactic acid, which allow to lowers the PH and creates an environment unfavorable to pathogens and spoilage organisms**. B (Nettle, 1993).** *Lactobacillus bulgaricus* and *streptococcus thermophiles* are the most important in genera of lactic acid bacteria it's involved to many dairy product such as cheese, yoghurt. Milk is a complete food, containing proteins, fats, carbohydrates, vitamins and mineral salts (Park et al., 2007), it's widely used for home consumption worldwide and to produce different cheeses and yoghurts **(cherigane and al 2007).** The objectives of this study were to collect a variety of samples milk in tow region Relizane and Adrar located in Algeria to determinate the thermophile species and their technological characters.

MATERIAL AND METHOD

Microbiological Analysis

Samples

A total of 20 samples of different row milk (sheep, cow, camel and goat) were collected from tow region Relizane and Adrar located in Algeria. The samples were immediately cools and transported in icebox with 4 degree C° and analyzed of content of laboratory on the arrival

Table 1: Media and Condition for Enumeration and Isolation of LAB

Microorganism	Media	T(C°)	Duration	Condition
Thermophilic Lactobacilli	MRS cetic(De Man et al., 1960) addition acetic acid	42	24-36	Anerobiosis
Streptococcus or *Enterococcus*	LM17(Terzaghi and Sandine,1975) Addition 5g of lactose.	42	24-72	Aerobiosis

Enumeration and Isolation the Thermophile Species

Ten (10ml) of each samples of raw milk homogenized with 90 ml of saline water in order to make initial dilution ($10^{-1)}$. The suspension was used for making suitable serial dilution up to 10^{-8} by incorporating 1ml in 9ml of sterile saline water in sterile tubes. The enumeration was determined by using two selective media such as MRS for the bacilli and M17 for cocci Agar as indicate de table 1. After incubation, colonies were enumerated, recorded as colony forming units (UFC) per liter of milk. The colonies were randomly picked and transferred in 10ml appropriate broth. The purity of isolates was checked by streaking again to fresh agar followed by macroscopic and microscopic examinations. The strains displaying characteristic lactic acid bacteria and considered as presumptive *Lactobacillus sp*in MRS and as presumptive *Streptococcus* or *Enterococcus* sp in M17 were chosen in order to use in further studies. The conservation for long time of isolates purified, without appreciable loss of properties was carried out to the medium containing70%skim milk (enriched by 0.05% of yeast extract and 0.05% of glucose) and 30% glycerol the isolates were stored at -80C°.working cultures were also kept in MRS or M17 agar slant at 4C°, renewed every 4weeks. **(Samelis et al., 1994; Herrero et al., 1996)**. According to many methods of characterization of isolates recommended by several authors such as **(Harrigan and McCance, 1976; Sharpe, 1979; Schleifer et al., 1985; Kandler and Weiss, 1986).** The isolates were gram stained, and tested of catalase production. Preliminary isolation and grouping on was basis of morphological and phenotypic properties using gas production from glucose, determined in M17 or MRS broth containing inverted Durham; growth at different temperature (10,15, and 45C°) and PH 9,6 as well as the ability to grow in different concentration such as (2%, 4% and 6.5%.w/v). Sherman test and survival after heating at 60C° for 30min **(Samelis et al., 1994)**; hydrolysis of arginine tested on MRS or M17 broth with bromocresol purple **(Thomas, 1973)** and production of acetone from glucose, determined by using the Voges-Prokauer test **(Zourari et al., 1991).**

Identification of Lactic Acid Bacteria to the Species Level

The fermentation of carbohydrate was determined according to the method described by **(Schillinger and Lücke (1987)** in MRS broth (without sugars) containing 1% of solution carbohydrate testing and added 0.025% bromocrsol purple as PH indicator. The carbohydrate tested were cellobiose, Galactose, Mannitol, melizitose, melibiose, ribose, trehalose, xylose, glucose, lactose, saccharose, fructose and arabinose. To ensure to anaerobic conditions, each tubes was topped up with two drops of sterile liquid paraffin after Incubation **(Samelis et al., 1994)**. the result was recorded after 48h

of incubation at 42C°. The fermentation patterns among carbohydrates was determined using the API20strep and API50 CHL gallery with API50CHL medium (bio merieux, Marcy-l'Etoil. France). The pib win database was used to interpret the results.

Properties Technologic

Acidification Activity of Isolates

The production of acid lactic by our isolates lactic acid bacteria species was determined after growing the isolates in MRS acetic (rods) and LM17 (cocci), and then inoculating in sterile reconstituted skim milk supplemented yeast extract (3g/l) and glucose (2g/l).sterile reconstituted skim milk was inoculated with 1% of overnight culture according to method descripted by **(Attia *et al.* (2001).** The inoculated culture was incubated at 42° for tow type thermophile isolates **(Farah *et al.*, 1990; De Vuyst and Degeest, 1999; Attia *et al.*, 2001).** change PH was mentioned at different intervals by tacking samples at 0h (initial),(6h),(12h),(24h),(72h) until the PH reached 4.6 (iso-electric point),as suggested by **Patrignani *et al*. (2007).** the isolated lactic acid bacteria species were considered as fast acid producers where the less than 12h to reach PH4.6, medium acid producers (12h,48h to reach 4.6) slow acid producers (more than 48h to reach PH4.6).

Evaluation of Proteolytic Activity

To evaluate proteolytic activity, The milk agar was prepared by adding 1% skim milk powder to Plate Count Agar (Himedia) (**Beerens & Luquet, 1990).** The surface dried milk agar was streaked bay culture overnight. The plates were incubated in 45C° in 48h.the transparent forming halo colonies were considered positive

Sttistical Analyses

Difference in acid production potential between the isolated lactic acid bacteria was determined by the analysis of variance technique using SPSS software (version 10) and Duncan Multiple Range test was used for mean separation when ANOVA showed statistical difference between means. Statistical differences were declared at 5% ($P< 0.05$) significance level **(Steel and Torrie, 1980).**

Antibacterial Activity

The inhibitory effect of stains LAB isolated and identified such as LB1, LB2, LB3 and ST1, E1, E2. Over strains pathogens was tested using two method direct and indirect: the first was the spot agar test (direct method) as carried by **Tagg and McGiven (1971)**and the second was the agar well diffusion assay as carried by **Schlillnger and Luck (1989)**using indicators bacteria pathogen such as *E. coli (ATCC* 25955, *Listeria monocytogenes (ATCC 7659) Staphylococcus aureus (ATCC 25925).*Were grown in bouillon nutritive (BN). For the direct method our strains were spotted in MRS Agar for the strains LB1.LB2 LB3 and M17 agar for the strains ST1, E1, E2 and incubated at 42C° for 18h.100µl of each culture indicator bacteria precedent were transferred in 10ml MH than poured over the spot and re-incubated at 42C°. The results were targeted by examination the zones of inhibition. For the indirect method : few colonies (4-5) were picked from each pathogen bacteria and suspended in 4ml of sterile water and standardized to approximately10^8CFU/ml in order to adjust standard turbidity to 0.5 of McFarland. A sterile swab were soaked in the suspension and inoculated in MHA (Muller Hinton Agar) plate. After the inoculum was added and allowed to absorb, and 6 mm sterile paper filter discs (Whatmann n°1) moistened with 20 ml of cell free supernatant obtained by centrifugation (2500 _ g/10 min) from each isolate of LAB in exponential growth phase were added. The susceptibility of pathogens to

the discs was assessed by measuring the zone of inhibition of bacterial growth around the discs (radius - mm) after incubation for 24 h at 37 _C. A clear zone of inhibition of at least 2 mm radius was recorded as positive. The experiment was performed in triplicate.

RESULTS AND DISCUSSIONS

Enumeration of Lactic Acid Bacteria

Table 2 summarizes the microbial count obtained from various samples The LAB was enumerated using selective media such as MRS and M17 from various samples. The presumptive *Streptococcus*sp or *Enterococcus sp* counts from $1x10^3$ cfu/ml for cow milk and from $2.3x10^3$ from sheep milk, a count $1.06x10^4$ cfu/ml from goat milk and finely, counts $7.2x10^3$cfu/ml from camel milk. For the presumptive *Lactobacillus* counts $39.5x10^3$ from cow milk and $17.2x10^3$ from sheep milk, counts to $7.8x10^3$ from goat milk, camel milk were counts $98.2x10^3$.we look that the *Streptococcus* or *Enterococcus* incubated at 42C° are less than *Lactobacillus* at 42 C°, and also, The LAB count to the camel milk is higher than other milk such as sheep milk **(Tornadijo et al., 1995; Badis et al., 2004).**

Table 2: Microbial Count from Various Samples in Cfu/Ml

Strains presumptive	Goat milk	Sheep milk	Cow milk	Camel milk
Streptococcus sp or *Enterococcussp*	$1.06x10^4$	$2.3x10^3$	$1x10^3$	$7.2x10^3$
Lactobacillus Thremophilic	$7.8x10^3$	$1.06x10^4$	$39.5x10^3$	$7.2x10^3$

Identification of LAB

In the present study, a total of 50 isolated of lactic acid bacteria thermophilic were obtained from different milk collected in region of Algeria. These isolates were identified to genus species level based on their cellular morphology, gas production, growth at 10C° and 45C°.In presence of 6.5% Nacl and PH 9.6 according to **wood and Holzapel (1995),**and biochemical test using API20 strep and API 50CHL. all the isolates are Gram positive, catalase negative and non-mobility and non-spores forming and thermophilic. The first screening revealed that the isolates were subdivided into 2 groups (1): 33 strains (14 isolates from ewe's milk, 9 isolates from camel milk, 2 isolates from sheep milk and 8 isolates from goat milk).these strains Were white, round or lenticular colonies cocci, diplococcicdiplococcic and in chine cells and homofermentative. Among to the isolates cocci; 23 isolates represented with tow specie, cocci were able to grow at 10C° and in NaCl 6.5% and PH 9.6 broth, and also survive at 60C° for 30min, capable to hydrolyze Arginine, able to grow the Sharman milk1%, hydrolyze of starch. 12 isolate capable to ferment starch, glucose, Lactose, Maltose, Mannitol, Mannose, Melibiose, Ribose, Sucrose, Sorbitol, Trehalose and variable for galactose and incapable to ferment Amygladin, arabinose, cellubiose, Fructose, Raffinose, Rhamnose, Xylose were characterized as *Enterococcus faecalis.* 17 isolates to form, Glucose, Lactose, Galactose, Maltose, Sucrose, Arabinose, Maltose, Mannitol, Trehalose, Cellubiose and didn't ferment Sorbitol, Raffinose, Xylose, Melibiose, Rhamonose were characterized as *Enterococcus faceium.* thelast four 04 isolates were unable to grow at 10C°,at PH 9.6.in addition, incapable to hydrolyze amidon and hydrolyze esculine and Sharman test 1% they did not survive in 60 C° for 30min. incapables to grew at 6.5% and 4%.for they formed acid from Glucose, Saccharose, Lactose and Galactose and variable for Raffinose and unable to ferment maltose, Mannitol, Trehalose, cellubiose, melebiose, Raffinose, Xylose, Ssucrose, arabinose, Melezitose, Rhamonose, Mannitol were characterized as

*streptococcus thermophilus***(Schleifer and Kilpper-Balz, 1984; Devriese et al., 1991;Manero and Blanch 1999).** The second group comprise: 17 isolates were classified into 3species of grams positive, catalase negative, rods, homofermentative, all isolates able to grow at 45C°. These characteristics suggest their classified as thermophilic facultative homofermentative *Lactobacillus* Among the isolates rods, 05 isolates considered as. *Lactobacillus delbrueckii subsp. bulgaricus* belong to their inability to hydrolyze Arginine and Esculine, and also incapable to grow in 6.5 % 2%, 4% of Na Cl. These isolates able to form acid fructose, glucose, lactose and didn't fermented Arabinose, Cellubiose, Galctose, Raffinose, Xylose. 03 isolates were able to grow at 2% of NaCl, variable to hydrolyze Arginine, able to form acid from sugars Galactose, Fructose, Glucose. Were characterize as *Lactobacillus delbrueckii subsp. lactis* and unable to Ferment sugars such as starch, Arabinose, Cellbiose, Mannose,Mellibiose,xylose.02 isolate Capable to from acid from the sugars Fructose, Glucose, Lactose, Maltose, Mannose, Sucrose. These isolates didn't fermented starch, Arabionose, Amygladin, Cellubiose, Galagtose, Mannitol, and were characterized as *Lactobacillus delbrueckiisubsp.delbrueckii*. The last 07 isolates considered as *Lactobacillus caseisubsp.rhamnosus* were characterized by fermentation the special sugars rhamnose and also the Majority of other sugars were fermented. The following table number 3 describes all characteristics physiological and biochemical of isolates.

Tabe 3: Physiological and Biochemical Characteristics of LAB Isolated From Various Raw Milk

Identified as	Thermophilicrods.				Thermophiliccocci		
	Lacobacillus delbrueckii Subsp bulgaricus N=05	*Lactobacillus delbrueckii subsp. Lactis N=03*	*Lactobacillus delbrueckii subsp. delbrueckii N=02*	*Lactobacillus caseisubsp rhamnosus N= 07*	*Enterococci faceium N=17*	*Enterococci faecalis N=12*	*Streptococcus thermophilus N=04*
Gram	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-
Morphology	Rods	Rods	Rods	Cocci	Cocci	Cocci	Cocci
Production of $CO_2$from Glucose	-	+	-	-	-	-	-
Growth at PH 9.6	-	+	-	-	-	-	-
Growth at	-	-	-	-	-	-	-
10C°	+	-	-	-	-	-	-
45C°	+	-	-	-	-	-	-
60C°from 30Min	-	+	-	-	-	-	-
NaCl							
2%	-	+	-	-	-	-	-
4%	-	-	-	-	-		-
6.5%	-	-	-	-	-	-	-
Sharman test (1%)	-	-	-	-	-	-	-
Hydrolyse of arginine (ADH)	-	-	-	-	-	-	-
Acetoin (VP)	-	-	-	-	-	-	-
Hydrolyse Amidon	-	-	-	-	-	-	-
Hydrolyse Esculine	-	-			-	-	-
Acid From							
Starch	-	-	-	-	-		-
Amygladin	-	-	-	-	-	-	-
Melizitose	-	-			-	-	-
Arabinose	-	-	–	–		-	
Cellubiose	-		–	–	–		–
Fructose	-	–	–	–	–	–	–
Galactose	-	+	+	+	–	–	–
Glucose	-	–			+	–	+
Lactose	-	–	–	–		+	
Maltose	-	+	+	+	–		–
Mannitol	-	V	+	+	+	–	+
Mannose	-	+			+	+	+
Melibiose	-	+	–	–		+	
Raffinose	-		+	+	–		–
Rhamnose	-	–			+	–	+
Ribose	-	+	–	–		+	
sucrose	+	–	–	–	–	–	–
Salicin	-	–	–	–	–	–	–
Sorbitol	+	–	–	–	–	–	–
Trealose;	+	–	+	+	–	–	–
Xylose	-	–	–	–	+	–	+
		-	-	-	-	+	-
+ : positive Reaction	- : negativeReaction				V:Variable Reaction		

Acidification Ability

The obtained results of acid production about 7 strains revealed that three LB1.LB2.LB3, can be characterized as the best fast acid producers belong to reduce the PH of the skim milk medium from an initial PH 6.71,7.11 and 7.20 to a final value of 4.6.4.38,4.73 respectively(figure1) with in 4h and a final titratable acidity value 70D°,75D°,69D° respectively with the same period (figure2). Whereas, one strain ST_1 considered as medium acid producer belong to the drop their PH from an initial value 6.77, to a final PH 4.6 within 48h (figure2).they also attained the titratable acidity 56D° at 48h of incubation. The *Enterococcus* species ; E1,E2 showed a slow acid production ability as it reduced the initial PH (6.77,6.56) to a final PH 4.53,4.33 about 72h of fermentation and attained final titratable acidity values of 43D°,49D° (figure2). These results are similar to that reported by authors such as **Hassaïne *et al*. (2007)**who indicated that the *Enterococcus* did not reducer the PH of milk to PH 5 after 24 h incubation. The decreasing of the PH during the process of fermentation of our strains has benefices such as prevention and inhibition for pathogenic flora **(Hassaïne *et al*. (2007).**

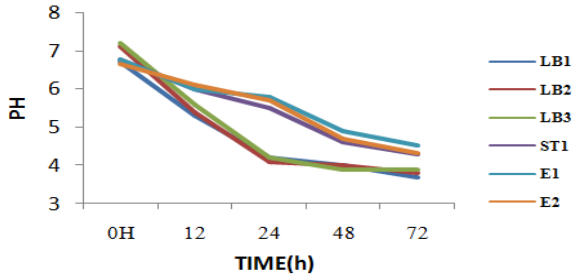

Figure 1: Change The Ph In Skim Milk of Lactic Acid Bacteria Acidity Incubated In 42C°.
(LB1:*Lacobacillus delbrueckii subsp Bulgaricus,* LB2*: Lactobacillus Delbrueckii Subsp. Lactis.*
LB3 *: Lactobacillus Casei subsp rhamnosus.* ST1 *: Streptococcus Thermophilus* E1:*Enterococcus Faecalis, E2:Enterococcusfaceium)*

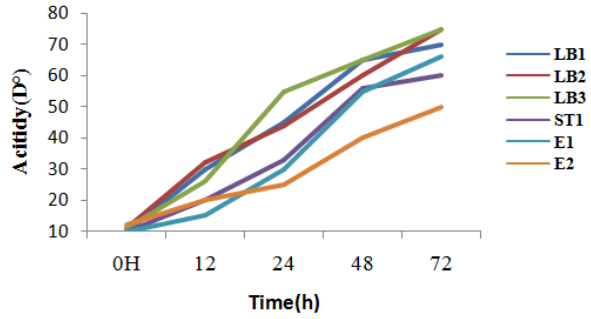

Figure 2: Evolution for Acidity En (D°) in Skim Milk of Lactic Acid Bacteria Acidity Incubated in 42C°.
(LB1:*Lacobacillus delbrueckii subsp Bulgaricus,* LB2*: Lactobacillus Delbrueckii Subsp. Lactis* .
LB3: *Lactobacillus Caseisubsprhamnosus.* ST1: *Streptococcus Thermophilus*
E1: Enterococcus*. Faecalis,* E2:*Enterococcu sfaeceium*

Activity Proteolytic:

The protelytic activity is very important factor in the development organoleptic properties of different dairy products. Proteolysis could also contribute to preventing allergies frequent in Children less than 3 years of age due to poor digestion of milk proteins **(Pescuma et al., 2009).** The production of good quality fermented dairy products is dependent on proteolytic properties of the starter bacteria, since peptidases and amino acids formed during fermentation have a direct impact on flavour development, or serve as flavour precursors in dairy products**. (EyassuSeifu and all, 2012).** In my test

all strains characterized as positive protelytic except the strain E2 (Table 3) with different diameter.the *Lactobacillus* considered as higher quality compared with *streptococcus* an *Enterococcus.*

Antimicrobial Activity

The results of antimicrobial activity were shown in figure3.the tested isolated indicated different levels of inhibitory action against of pathogenic strains.5 isolates were demonstrated an inhibitory activity against four pathogen strains. with different zone of inhibition. the most inhibition isolate was showing by the LB1 against *E coli*, *staphylococcus areus* and *Listeria monosytongne* with an inhibition zone 5.5 mm,4.5 mm 4.9 respectively suited by St2 with 4 mm 3mm, 3.8mm respectively and wake inhibition for *Enterococcus* with 2mm, 2.2mm for *Enterococcus faecalis*, negative for *Enterococcus faeceuim*. This results are similar than other authors like **(Washington LuizGonçalves and all 2015),** Where indicate the power of inhibition action of *Lactobacillus.*

Table 4: Activity Proteolytic of LAB with Evaluation of Zone of Hydrolyze

Strains	*E.coli*	*S.areus*	*L.M*	Evaluation
LB1	+++	+++	+++	Good
LB2	+++	+++	+++	Good
LB3	+++	+++	+++	Good
ST1	++	++	++	Medium
E1	+	+	+	Wake
E2	-	-	-	Negative

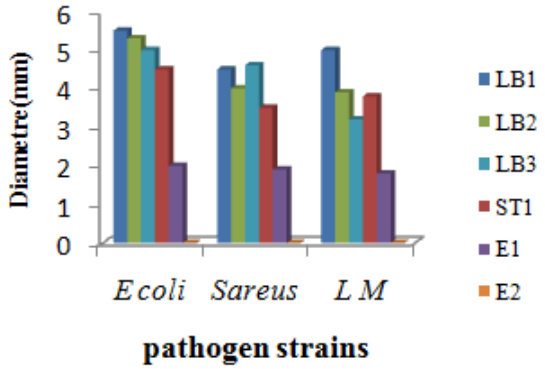

Figure 3 : LB1: ***Lactobacillus Delbrueckii subsp, Bulgaricus,*** **LB2*: Lactobacillus Delbrueckii subsp. Lactis*.**
LB3 ***: Lactobacillus Casei subsp rhamnosus.*** **ST1** ***: Streptococcus Thermophilus***
E1:*Enterococcus. Faecalis*e2:*Enterococcusfaceium*.

CONCLUSIONS

Our study revealed that dairy milk of Algeria has high potential of lactic acid bacteria including in various fermentation. The dominants termophilic acid lactic bacteria in various fermented milk were *Lactobacillus* (10%), *Enterococcus (58%), and Streptococcus* (8%). in order to use as starter cultures in manufactures fermented milk, we should to focus in future research for desirable characteristics such as acidity, production exopolysaccharide of bacteriocins and aroma. Many studies confirmed that the ability of LAB to inhibit the other strains pathogen in dairy products with beneficial effects on human health.

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